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The prognostic utility of haptoglobin genotypes in squamous cell carcinoma of the head and neck

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Abstract

Background: The aim of this study was to determine whether haptoglobin (Hp) genotypes are associated with prognosis in patients with squamous cell carcinoma of the head and neck (HNSCC).
Methods: We studied patients with HNSCC without distant metastasis at diagnosis. The Hp genotype of each patient was determined and the prognostic significance of the Hp genotype was further analyzed. Pearson's χ^2-test or Fisher's exact test were used to analyze correlations between Hp genotype and clinical characteristics of HNSCC. Eighty patients with newly diagnosed HNSCC who were treated with curative modality were enrolled in this study. Kaplan-Meier plots and log-rank test were used to compare locoregional recurrence-free survival, distant-metastasis-free survival and overall survival of patients according to Hp genotype. Survival analysis was performed using Cox proportional hazard models.
Results: Eighty patients with newly diagnosed HNSCC were enrolled in this study. There was no significant difference in the distribution of Hp genotypes in HNSCC patients and healthy individuals ($p=0.959$). Matched-pair analysis showed that locoregional recurrence-free survival was poor ($p=0.02$) for HNSCC patients with Hp 2-2 or 2-0. There was no significant difference in distant metastasis-free survival and overall survival ($p=0.422$ and 0.509, respectively). Multivariate analysis showed that Hp 2-2 or 2-0 was associated with an increased risk of locoregional recurrence [Hazard ratio (HR) 5.9; 95% confidence interval (CI), 1.1–6.65; $p=0.038$]. The risk was still higher in patients with Hp 2-2 or 2-0 after further adjusting for age and treatment modality (HR 7.6; 95% CI, 1.2–46; $p=0.028$) in locoregional recurrence-free survival.
Conclusions: The present data show that the Hp genotype is closely related to recurrence rate in patients with HNSCC. Patients with Hp 2-2 or 2-0 have greater locoregional recurrence and significantly increased HRs in multivariate analysis. The Hp genotype may be a prognostic factor in patients with HNSCC.
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Keywords: genotype; haptoglobin; head and neck squamous cell carcinoma; survival.

Introduction

Squamous cell carcinoma of the head and neck (HNSCC) is one of the most common cancers worldwide that occurs predominantly in middle-aged and older men who use tobacco, alcohol, and betel nuts. In Taiwan, the incidence of HNSCC has continued to increase and is currently the fourth most common cause of cancer-related mortality in men (1). HNSCC has a high recurrence rate and poor survival. The prognosis and clinical outcomes of patients with HNSCC depends primarily on the TNM staging system. However, the most popular and widely used TNM system does not always predict prognosis well. Reliable prognostic factors may be used to supplement the present staging system.

Haptoglobin (Hp), a hepatocyte-derived serum α_2-glycoprotein, is an acute-phase reactant produced in response to inflammatory cytokines and glucocorticosteroids (2, 3). Previous studies have shown that Hp may play an important role in host defense against infection and neoplasms by decreasing the availability of iron to invading pathogens and tumor cells, and by modulating angiogenesis and other inflammatory processes (4, 5). The expression of Hp is controlled by the polymorphic Hp locus located on chromosome 16q22. The characteristics of Hp vary according to the different Hp genotypes. Hp 1-1 is a much better antioxidant compared with HP 2-2 or HP 2-0, and binds more strongly with free hemoglobin (3, 6, 7). Angiogenic effects are more frequently observed with Hp 2-2, while Hp 2-1 has an intermediate characteristic (8, 9).

Several studies have investigated the relationship between Hp concentrations and head and neck cancer (10, 11). However, there are no published data on the

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prognostic influence of Hp genotypes in HNSCC. The goal of this study was to investigate the prognostic influence of Hp genotypes in HNSCC.

Materials and methods

Human subjects

The study was conducted with the approval of the Human Research Ethics Committees of our institution. Written informed consent was obtained from all patients with HNSCC and healthy individuals between 2005 and 2008. A total of 92 cases were identified and retrieved from the archives. Twelve patients were not eligible for analyses because of the presence of distant metastasis at the time of presentation and loss of follow-up. In this study, healthy subjects, who were asymptomatic and underwent physical examination, chest X-ray, abdominal sonogram for reasons of promoting good health and disease prevention during annual health check-up were recruited as control subjects. All blood samples were collected in EDTA-containing tubes. A total of 80 HNSCC patients for whom complete data sets were available were analyzed. The following data were recorded for each patient: age, gender, tobacco use, alcohol use, betel nut chewing, treatment modality, and outcome. All tumors were staged according to the American Joint Committee on Cancer (AJCC) system, modified in 2002.

All patients initially received curative treatment. On completion of treatment, patients were followed regularly every month during the first year, every 2 months in the second year and every 3 months thereafter. Chest X-rays were performed annually, while head and neck magnetic resonance imaging/computed tomography, bone scans and ultrasound of abdomen were performed when clinically indicated.

Hp genotyping based on polymerase chain reaction (PCR)

Genomic DNA was extracted from peripheral blood mononuclear cells using a DNA extraction kit (Qiagen, Valencia, CA, USA). PCR methods were used to analyze the Hp 1 and Hp 2 alleles as described previously (12, 13). Primers Hp-del-U and Hp-del-L were used for amplification of a 315-base pair (bp) Hp 0 allele-specific sequence. Primers Hp-exon-U and Hp-exon-L were used to amplify a 476-bp Hp 1 or Hp 2 allele-specific sequence (14, 15). PCR was carried out in a final volume of 50 μL containing 10–20 ng of genomic DNA. For protocol 1, 1× PCR buffer (Qiagen), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 μM of each primer, and 2 units of Qiagen *tag* DNA polymerase were included in the PCR reactions using primers A and B. The program for PCR using primers A and B was as follows: an initial incubation at 95°C for 5 min, 35 cycles of incubation at 95°C for 1 min, 66°C for 40 s, and 72°C for 90 s, with a final extension at 72°C for 10 min. For protocol 2, 1× PCR buffer (Qiagen), 1.5 mM $MgCl_2$, 0.05 mM dNTPs, 0.2 μM of each primer, and 2 units of ABgene *tag* DNA polymerase were included in the PCR reactions using primers C and D. For the PCR with primers C and D, the program was as follows: an initial incubation at 95°C for 5 min, 35 cycles of incubation at 95°C for 1 min, 69°C for 1 min, with a final extension at 72°C for 10 min. For PCR protocol 3, 1× PCR buffer (Qiagen), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM of each primer, and 2 units of Qiagen *taq* DNA polymerase were included in the PCR reactions. The thermal cycler conditions used for amplification were as follows: an initial incubation at 95°C for 5 min, 35 cycles of incubation at 95°C for 30 s, 60°C for 40 s, and 72°C for 1 min, with a final extension at 72°C for 10 min. The resulting PCR products underwent electrophoresis in 1% agarose gel stained with ethidium bromide. All patient samples were analyzed using protocol 1 with primers A and B. If the presence of the 1757-bp product was detected, the samples were later analyzed according to protocol 2 with primers C and D. The Hp 0-specific PCR product was detected as a 315-bp band, whereas the product of Hp exon 1 was detected as a 476-bp band. The sequences of primers were:
A: 5′-GAGGGGAGCTTGCCTTTCCATTG-3′
B: 5′-GAGATTTTTGAGCCCTGGCTGGT-3′
C: 5′-CCTGCCTCGTATTAACTGCACCAT-3′
D: 5′-CCGAGTGCTCCACATAGCCATGT-3′
Hp-del-U: 5′-CTTTATGGCACTGGGGAACAAGCATTTTG-3′
Hp-del-L: 5′-CAGGAAGAGATTTTTAGCCGTGGTCAGCAG-3′
Hp-exon-U: 5′-GCAGTGTGAAAATCCTCCAAGATAA-3′
Hp-exon-L: 5′-AATTTAGCCCATTTGCCCGTTTCTT-3′.

Analyzing survival rates with matched-pair method

In order to limit the effect of confounders and to increase statistical efficiency, a matched-pair method was conducted. Previous studies showed that patients with nasopharyngeal carcinoma and breast cancer who had the Hp 1-1/2-1 genotype had a better prognosis (16, 17). Due to these observations, we assigned HNSCC patients into two groups: patients with Hp 1-1 or 2-1 and patients with Hp 2-2 or 2-0. Patients with Hp 1-1 or 2-1 were matched randomly with patients with Hp 2-2 or 2-0 in a 1:1 ratio. Matching variables were gender, site of primary tumor, and the stage of clinical disease.

Statistical analysis

All data were analyzed using the SPSS (Version 12, SPSS Inc., Chicago, IL, USA) system. The association between different categorical variables was analyzed with Pearson's χ^2-test or Fisher's exact test. Hardy-Weinberg equilibrium analyses for Hp polymorphism were conducted with one degree of freedom in the HNSCC patients and controls. Survival rates were estimated using the Kaplan-Meier method and Kaplan-Meier survival curves were compared using the log-rank test. The Cox proportional regression model was used to evaluate the effect of Hp genotypes on survival rates after adjusting for matching variables and other potential prognostic factors.

Results

The following Hp genotype-specific banding was obtained: Hp 1-1 and Hp 2-2 were characterized by single bands representing the 1757- and 3481-bp products, respectively. Genotype 2-1 was characterized by the presence of both the 1757- and 3481-bp products (Figure 1A). With respect to Hp 2-1, the Hp 1-specific 1757-bp band was generally more intense than the Hp 2-specific 3481-bp band (Figure 1A, lane 2). Figure 1B shows that patients without the 349-bp product had the Hp 1-1 genotype (lane 5). Figure 1C shows the Hp 0 pattern.

Figure 2 shows patient enrollment in this study. A total of 80 patients with HNSCC with complete data were enrolled. Also, 80 healthy individuals of similar age and gender were included as a comparison group for the distribution of Hp genotypes. The final study sample of matched-pair analysis consisted of 46

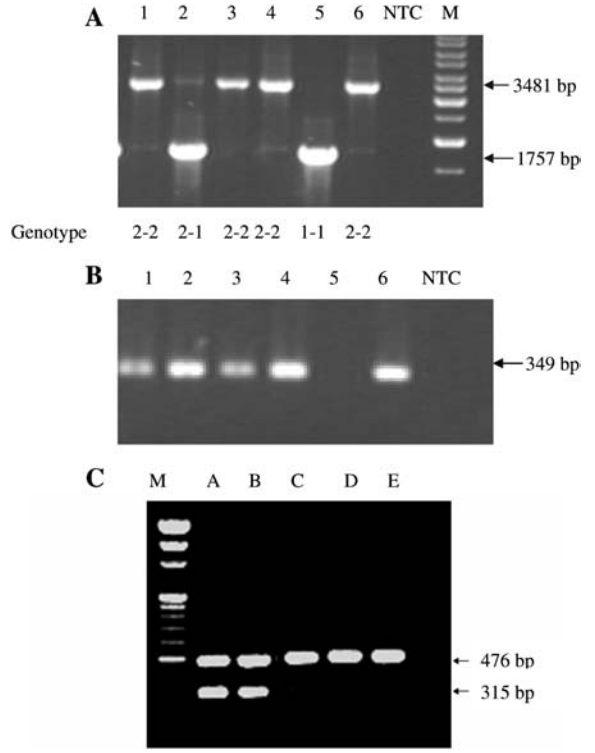

Figure 1 Haptoglobin genotyping. Agarose gels demonstrating genotype determinations using DNA from individuals representing genotype Hp 1-1, Hp 2-1 and Hp 2-2.
(A) Haptoglobin genotyping with primer A and B (protocol 1). (B) Protocol 2 with primer C and D was used to determine the complete genotype when the 1757-bp band was detected. A 349-bp product was generated from the genomic DNA of individuals homozygous or heterozygous for the Hp 2 allele, whereas no product was formed in the presence of the Hp 1 allele (lane 5, DNA from the individuals with the Hp 1-1 genotype). (C) Detection of haptoglobin gene deletion. Both 315-bp and 476-bp bands were amplified from individuals heterozygous for the Hp 0 allele (lanes A and B).

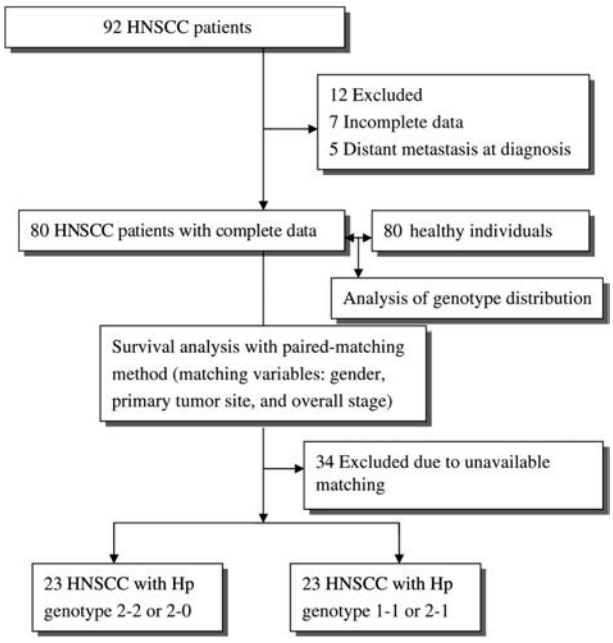

Figure 2 Flow diagram for analysis of genotype distribution and survival analysis with paired-matching method.

patients with HNSCC (44 men, 2 women; mean age, 51 years), 23 patients with Hp 1-1 or 2-1 and 23 patients with Hp 2-1 or Hp 2-0. In each group, there were 11 patients with oral cancer, four patients with oropharyngeal cancer, and eight patients with hypopharyngeal cancer.

Association between HNSCC and Hp genotypes

The first part of the study was comprised of 80 HNSCC patients and 80 healthy individuals. Table 1 shows the distribution of Hp genotypes in healthy individuals and patients with HNSCC. There was no significant difference in the distribution of Hp genotypes between these two groups ($p=0.959$). The distribution of genotypes in healthy individuals and HNSCC patients were in Hardy-Weinberg equilibrium ($p=0.139$ and 0.113, respectively).

Association between Hp genotypes and survival of HNSCC patients

The demographics of the two groups were similar. There were no significant differences between the two populations in terms of gender, tobacco use, alcohol use and betel nut chewing. The patients were evenly matched with respect to tumor stage, tumor classification, and nodal status (Table 2). Although the two groups were not matched with respect to treatment modality, the types of treatment received were similar.

The time of follow-up ranged from 5 to 60 months [mean $\pm$ standard deviation (SD), 21 ± 11.7 months; median, 21 months] for the HNSCC patients with Hp 1-1 or 2-1, and ranged from 4 to 52 months (mean $\pm$ SD, 20 ± 10.2 months; median, 19 months) for the HNSCC patients with Hp 2-2 or 2-0. HNSCC

Table 1 Hp genotype distribution in the normal control group and HNSCC patients.

Variables	Control group (n=80)	HNSCC group (n=80)	p-Value
Gender			
Male	78 (69)	78(69)	
Female	2 (31)	2 (31)	
Age			0.652
Mean $\pm$ SD	51 ± 12	52 ± 11	
Genotype			0.959
Hp 1-1	8 (10)	8(10)	
Hp 2-1	35 (44)	32 (40)	
Hp 2-2	33 (41)	35 (44)	
Hp 2-0	4 (5)	5 (6)	
$\chi^{2\,a,b}$	2.179	2.5	
Alle frequency			
Hp 1	0.319	0.3	
Hp 2	0.656	0.669	
Hp 0	0.025	0.031	

Values are given as number (percentage). [a]χ^2-test for deviation from Hardy-Weinberg equilibrium; [a]p-values from goodness-of-fit χ^2-test in Hardy-Weinberg equilibrium in the control group was 0.139, and 0.113 in the HNSCC group. HNSCC, head and neck squamous cell carcinoma; SD, standard deviation; Hp, haptoglobin.

Table 2 Characteristics of patients: matching variables.

	Genotype		p-Value
	Hp 1-1, 2-1 (n=23)[a]	Hp 2-2, 2-0 (n=23)[b]	
Gender			
Male	22 (96)	22 (96)	
Female	1 (4)	1 (4)	
Age in years			
Mean±SD	52±12	50±11	0.739
Primary site			
Oral cavity	11	11	
Oropharynx	4	4	
Hypopharynx	8	8	
Stage			
III	1 (4)	1 (4)	
IV	22 (96)	22 (96)	
T classification			0.562
T2	7 (30)	4 (17)	
T3-4	16 (70)	19 (83)	
N classification			1
N0-1	7 (30)	7 (30)	
N2-3	16 (70)	16 (70)	
Treatment			0.475
Surgery+adjuvant R/T or adjuvant CCRT	17 (74)	19 (83)	
CCRT	6 (26)	4 (17)	

Values are given as number (percentage). [a]Five patients with Hp 1-1 genotype and 18 patients with Hp 2-1; [b]20 patients with Hp 2-2 genotype and three patients with Hp 2-0 genotype. Hp, haptoglobin; SD, standard deviation; R/T, radiotherapy; CCRT, concurrent chemoradiotherapy.

patients with Hp 1-1 or 2-1 had better locoregional recurrence-free survival rates (p=0.02; Figure 3A). Table 3 shows the failure patterns of HNSCC patients with different Hp genotypes. Ten of 23 (44%) HNSCC patients with Hp 2-2 or 2-0 had locoregional recurrence, vs. three of 23 (13%) HNSCC patients with Hp 1-1 or 2-1 (p=0.022). Patients with recurrent tumor received salvage treatment. Seven of 10 HNSCC patients with Hp 2-2 or 2-0 received salvage surgery with or without adjuvant chemoradiotherapy and four patients were successfully salvaged; three patients received concurrent chemoradiotherapy for their recurrent tumor and one patient was successfully salvaged. Two of three HNSCC patients with Hp 1-1 or 2-1 received surgical intervention and neither of these patients were successfully salvaged; one patient who received chemotherapy was successfully salvaged. There was no significant difference with respect to distant metastasis-free survival and overall survival rates (p=0.422 and 0.509, respectively). Five of 23 (23%) HNSCC patients with Hp 2-2 or 2-0 had distant metastasis, vs. seven of 23 (30%) HNSCC patients with Hp 1-1 or 2-1 (p=0.559); eight of 23 (35%) patients with Hp 2-2 or 2-0 died, vs. nine of 23 (39%) patients with Hp 1-1 or 2-1 (p=0.76). Patients with distant metastasis received palliative treatment. There was a significant difference in terms of risk of locoregional recurrence-free survival between these two groups after adjusting for gender, tumor site, and stage [Hazard ratio (HR) 5.9; 95% confidence interval (CI) 1.1–6.65; p=0.038; Table 4]. The risk was still higher for the patients with Hp 2-2 or 2-0 after further adjusting for age and treatment modality (HR 7.6; 95% CI, 1.2–46; p=0.028) with respect to locoregional recurrence-free survival. The HR was not significantly increased in distant metastasis-free survival and overall survival (p=0.339 and 0.776, respectively; Table 4).

Discussion

The genotypes of Hp can be readily determined (12–15) using PCR and electrophoresis. Our data demonstrated that the pattern of Hp genotype distribution in patients with HNSCC was not different from that of healthy individuals. The distribution of Hp genotypes in healthy individuals and patients with HNSCC was in Hardy-Weinberg equilibrium. Although previous studies showed the relationship between specific Hp genotypes and cancer, this phenomenon was not observed in HNSCC (3).

Our previous study showed that Hp 2-2 was a negative prognostic factor for nasopharyngeal carcinoma (16). Gast et al. also reported that Hp 2-2 was associated with poor survival in breast cancer patients (17). We divided our HNSCC patients into two groups because of these studies. With matched-pair analysis, 23 patients with Hp 2-2 or 2-0 were pair-matched by gender, primary tumor site, and stage to 23 patients with Hp 1-1 or 2-1. The patient demographics presented in Table 1 show that the two groups were similar. The two populations could be compared with respect to recurrence, distant metastasis, and overall survival. Figure 3 shows that HNSCC patients with Hp 2-2 or 2-0 had poor locoregional recurrence survival rates (p=0.02). Using gender, primary tumor site, and overall stage as covariates, Hp 2-2 or 2-0 was an independent prognostic factor for locoregional recurrence (HR=5.9; p=0.038). The risk was still higher in patients with Hp 2-2 or 2-0 after further adjusting for age and treatment modality (HR 7.6; p=0.028) with respect to locoregional recurrence-free survival. Although there was greater recurrence in HNSCC patients with Hp 2-2 or 2-0, the survival rates were not statistically significant. Seven of 10 (70%) recurrent HNSCC patients with Hp 2-2 or 2-0 underwent salvage surgery and adjuvant therapy and three patients received salvage chemoradiotherapy. In the Hp 2-2/2-0 group, five HNSCC patients with recurrent tumor were eventually successfully salvaged. This may be one of the reasons why there was no significant difference in survival rates between these two groups.

Some studies investigated the relationship between Hp concentrations and both HNSCC and nasopharyngeal carcinoma (10, 11). Free hemoglobin promotes the accumulation of free radicals and other harmful reactive oxygen species, as well as the breakdown of erythrocytes in interstitial fluid resulting in hemoglobin-mediated free radical formation (18). Plasma Hp is a major antioxidant and can protect against hemoglobin-mediated lipid peroxidation (19). Lim et al. (20) showed that in Hp knockout mice, more deaths and renal damage occurred during hemolysis. The refer-

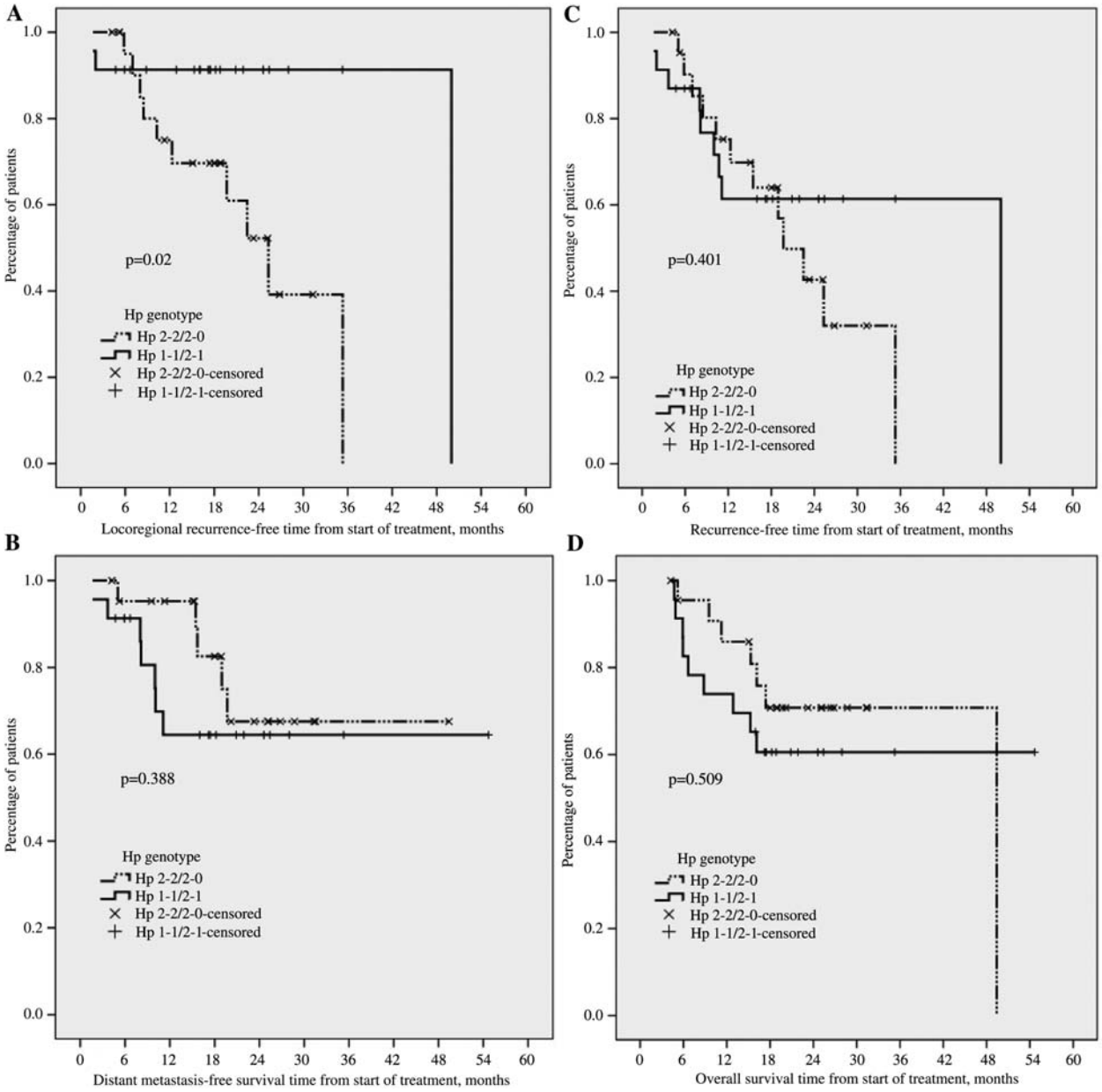

Figure 3 Survival curves of different haptoglobin genotypes.
(A) Locoregional recurrence-free survival; (B) distant metastasis-free survival; (C) recurrence-free survival and (D) overall survival in patients with head and neck squamous cell carcinoma with different haptoglobin genotypes.

ence range for Hp is lower in individuals with Hp 2-2 and this may result in reduced protection against free radicals (6, 21). The antioxidative capacity of body fluids is less efficient in Hp 2-2 individuals. This finding was attributed to the fact that the Hp 2-2 phenotype produces higher molecular mass species (>200 kDa) which restrict their distribution in interstitial fluid (6). Tseng et al. (7) reported that the ranking of the antioxidant activity was as follows: Hp 1-1>Hp 2-1>Hp 2-2 in an vitro study. Also, transfected Chinese ovary cell (CHO) with Hp 1-1 expression had increased tolerance against oxidative stress than CHO cells expressing other Hp genotypes. Furthermore, individuals with Hp 2-0 have extremely low concentrations of Hp (14). Greater locoregional recurrence rates in HNSCC patients with Hp 2-2 or 2-0 may be partially attributed to the poor antioxidant capacity of Hp 2-2 and 2-0 type when compared with other Hp types. Individuals with a Hp 2-2 or 2-0 genotype have lower Hp concentrations and therefore, less efficient hemoglobin-binding capacity. This results in some degree of heme iron accumulation as shown by the association of Hp 2-2 with higher serum iron, higher transferrin saturation, and higher serum ferritin concentrations (20, 22). Iron promotes neoplastic cell growth and accumulates in cancer cells more readily than in normal cells, and excess iron may suppress the tumoricidal activity of macrophages and interfere with lymphocyte trafficking (23, 24).

Hp is involved in arterial restructuring by facilitating cell migration through the accumulation of a temporary gelatin matrix. It has been suggested that Hp may be involved in other vascular and non-vascular processes such as angiogenesis and tumor cell invasion

Table 3 Failure patterns of patients with HNSCC according to Hp genotype.

	Genotype		p-Value
	Hp 1-1, 2-1 (n = 23)[a]	Hp 2-2, 2-0 (n = 23)[b]	
Failure patterns			
Locoregional	3 (13)	10 (44)	0.022
Distant metastasis	7 (30)	5 (23)	0.559
Locoregional and distant metastasis	1 (4)	1 (4)	1
Survival status			
Death	9 (39)	7 (30)	0.536

Values are given as number (percentage). [a]Five patients with Hp 1-1 genotype and 18 patients with Hp 2-1; [b]20 patients with Hp 2-2 genotype and three patients with Hp 2-0 genotype. HNSCC, head and neck squamous cell carcinoma; Hp, haptoglobin.

Table 4 HR of locoregional recurrence, distant metastasis and overall death from disease associated with Hp genotype 2-2 or 2-0 vs. Hp genotype 1-1 or 2-1 in head and neck cancer.

Analysis	Locoregional recurrence HR (95% CI)	p-Value	Distant metastasis HR (95% CI)	p-Value	Overall death HR (95% CI)	p-Value
Cox regression analysis on matching variables (gender, disease site, stage)	5.9 (1.104–6.65)	0.038	0.4 (0.121–1.465)	0.174	0.8 (0.296–2.385)	0.743
Adjusted for age and treatment modality	7.6 (1.2–46)	0.028	0.55 (0.157–1.894)	0.339	1.17 (0.416–3.285)	0.776

HR, hazard ratio; Hp, haptoglobin; 95% CI, 95% confidence interval.

(8). Hp 2-2 stimulates endothelial cell differentiation and has more angiogenic effects than other Hp phenotypes, both in in-vitro and in-vivo models of angiogenesis (9). Kuhajda et al. (25) showed that decreased tumor expression of Hp-related protein is associated with better recurrence-free survival in patients with breast cancer. The expression of a peak at mass-to-charge ratio 9198 > 20, identified as a Hp α-1 chain, has better recurrence free survival, and 63 breast cancer patients with Hp 2-2, which lacked an Hp α-1 chain, conferred a poor prognosis (17). Collectively, these observations may present a possible mechanism for free radical generation and cancer progression in patients with Hp 2-2.

There are several limitations of this study. First, the oxidative stress markers and serum Hp concentration were not measured. Second, the number of cases was limited. We tried to limit the effect of potential confounders by matching analysis. The matching for three variables known to affect prognosis (gender, tumor site, and overall stage) renders it more likely to detect any differences due to Hp polymorphism.

Patients with Hp 2-2 or 2-0 have more locoregional recurrences and significantly increased HRs in multivariate analysis. The Hp genotype may be a prognostic factor in HNSCC.

Conflict of interest

The authors have no relevant financial interest in this article.

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